

# THE NF- $\kappa$ B AND I $\kappa$ B PROTEINS: New Discoveries and Insights

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## ABSTRACT

The transcription factor NF- $\kappa$ B has attracted widespread attention among researchers in many fields based on the following: its unusual and rapid regulation, the wide range of genes that it controls, its central role in immunological processes, the complexity of its subunits, and its apparent involvement in several diseases. A primary level of control for NF- $\kappa$ B is through interactions with an inhibitor protein called I $\kappa$ B. Recent evidence confirms the existence of multiple forms of I $\kappa$ B that appear to regulate NF- $\kappa$ B by distinct mechanisms. NF- $\kappa$ B can be activated by exposure of cells to LPS or inflammatory cytokines such as TNF or IL-1, viral infection or expression of certain viral gene products, UV irradiation, B or T cell activation, and by other physiological and nonphysiological stimuli. Activation of NF- $\kappa$ B to move into the nucleus is controlled by the targeted phosphorylation and subsequent degradation of I $\kappa$ B. Exciting new research has elaborated several important and unexpected findings that explain mechanisms involved in the activation of NF- $\kappa$ B. In the nucleus, NF- $\kappa$ B dimers bind to target DNA elements and activate transcription of genes encoding proteins involved with immune or inflammation responses and with cell growth control. Recent data provide evidence that NF- $\kappa$ B is constitutively active in several cell types, potentially playing unexpected roles in regulation of gene expression. In addition to advances in describing the mechanisms of NF- $\kappa$ B activation, excitement in NF- $\kappa$ B research has been generated by the first report of a crystal structure for one form of NF- $\kappa$ B, the first gene knockout studies for different forms of NF- $\kappa$ B

and of I $\kappa$ B, and the implications for therapies of diseases thought to involve the inappropriate activation of NF- $\kappa$ B.

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## 1. INTRODUCTION

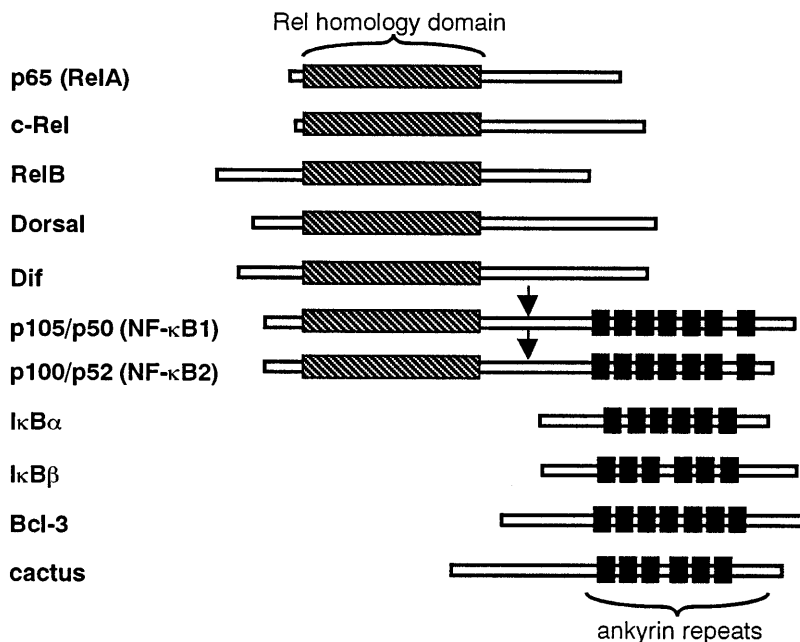
Ten years ago Sen & Baltimore (1) first described NF- $\kappa$ B as a B cell nuclear factor that bound a site in the immunoglobulin  $\kappa$  enhancer. In the same year, these researchers also showed (2) that NF- $\kappa$ B could be activated in other cells by exposure to stimuli such as phorbol esters, and that this activation was independent of protein synthesis. In the next few years, functional NF- $\kappa$ B binding sites were found in the promoters of many genes, most of which were not B cell specific. These promoter/enhancers included IL-2, IL-6, GM-CSF, ICAM-1, and class I MHC. Typically, NF- $\kappa$ B binding sites serve as inducible transcriptional regulatory elements that respond to immunological stimuli such as TNF, IL-1, LPS, or T cell activators. However, the range of inducers is not limited to these mediators of immune function; other stimuli such as UV irradiation, growth factors, and viral infection also activate NF- $\kappa$ B. The basis for the latent nature of NF- $\kappa$ B and for its inducibility is the association of NF- $\kappa$ B with a cytoplasmic inhibitory protein called I $\kappa$ B (3). The release from I $\kappa$ B allows for the extraordinarily rapid appearance of NF- $\kappa$ B in the nucleus. Thus, certain genes regulated by NF- $\kappa$ B can be transcriptionally activated within minutes following exposure to the relevant inducer.

Ten years after its discovery, the NF- $\kappa$ B and I $\kappa$ B field remains a lively arena for research. Five members of the mammalian NF- $\kappa$ B/Rel proteins have been identified that are characterized by the Rel homology domain (RHD), an N-terminal region of approximately 300 amino acids. These proteins are members of an evolutionarily conserved family of proteins, some of which regulate body pattern formation and immune function in insects. Consistent with a complex system for regulatory control, there are multiple forms of I $\kappa$ B proteins characterized by several copies of the so-called ankyrin repeat. Recent research has elaborated several of the critical aspects of signaling that mediate NF- $\kappa$ B translocation in response to inducer. Gene knockout studies firmly establish a role for NF- $\kappa$ B in immune function and eliminate any models that claim simple redundancy for the functions of the different NF- $\kappa$ B/Rel proteins. Regulation of NF- $\kappa$ B by the network of regulatory cytokines and other immune function modulators is now well established and is growing in complexity. Additionally, the gene deletion studies as well as other approaches provide support for a role of the NF- $\kappa$ B proteins in functions beyond immunity and inflammatory responses, including roles in liver development and in several disease processes.

Two recent *Annual Reviews* chapters (4, 5) have covered NF- $\kappa$ B in great detail through much of 1993. These and other reviews (6–12) offer a thorough background on NF- $\kappa$ B. The aim of this review is to provide coverage of some of the significant advances in NF- $\kappa$ B over the last two years. Many reports involving NF- $\kappa$ B have appeared during this time; due to space limitations, it is impossible for this review to cover all of these important results. In addition, reviews reference previous work where appropriate.

## 2. THE NF- $\kappa$ B/REL AND I $\kappa$ B PROTEINS

Presently, five members of the mammalian NF- $\kappa$ B/Rel family have been cloned and characterized. These are c-Rel, NF- $\kappa$ B1 (p50/p105), NF- $\kappa$ B2 (p52/p100), RelA (p65), and RelB (4–12). The Rel homology domain (RHD; see Figure 1) found in each of these proteins functions in DNA binding, dimerization, and interactions with I $\kappa$ B forms. Two of the proteins, NF- $\kappa$ B1 (p105) and NF- $\kappa$ B2 (p100), contain multiple copies of the so-called ankyrin repeat at their C-termini.



*Figure 1* The NF- $\kappa$ B/Rel and I $\kappa$ B families of proteins. The NF- $\kappa$ B/Rel family is characterized by the presence of the Rel homology domain. The I $\kappa$ B proteins have multiple copies of the ankyrin repeat. NF- $\kappa$ B1 and NF- $\kappa$ B2 are proteins that contain both the Rel homology domain and ankyrin repeats. Dorsal, Dif, and Cactus are *Drosophila* proteins. See text for discussion.

Processing of these proteins (see below) leads to the production of the p50 and p52 subunits. The p100 and p105 proteins serve regulatory functions in the cell (see below) and should not be considered exclusively as precursor forms. Furthermore, some evidence suggests that alternative splicing of the p105 mRNA leads to the translation of additional forms of this protein (13).

## 2.1 *NF- $\kappa$ B Is a Dimer of Variable Subunits*

Active, DNA-binding NF- $\kappa$ B is a dimer. Classic NF- $\kappa$ B (the dimer of p50 and RelA) has been the most intensively studied, although many other homo- and heterodimers have been described. Certain dimers apparently do not exist; for example, RelB dimerizes only with p50 or p52 (14). Each of the various NF- $\kappa$ B dimers may exhibit distinct properties. For example, binding site preference has been identified for certain dimers. Classic NF- $\kappa$ B binds the sequence 5' GGGRNYYCC 3', whereas the RelA/c-Rel dimer binds to a sequence (5' HGGARNYYCC 3'; H indicates A, C or T; R is purine; Y is pyrimidine) (15). Selective binding sites for RelA/c-Rel heterodimers are found in the promoters of several inducible genes, including those encoding tissue factor and GM-CSF (15); the RelA/c-Rel dimer is inducible by LPS or by cytokines. The ability of different dimers to recognize slightly different DNA targets increases the ability of NF- $\kappa$ B subunits to differentially regulate gene expression. More examples of biologically relevant targets for the different dimers and of possible roles in gene-specific transcription are needed. Additional differences between the NF- $\kappa$ B dimers include cell type specificity, differential subcellular localization, differential interactions with forms of I $\kappa$ B, and differential activation (4, 5).

## 2.2 *Dimers of NF- $\kappa$ B Are Targeted by Monomers of I $\kappa$ B*

Initial characterization of cytoplasmic NF- $\kappa$ B revealed that it was associated with either of two forms of I $\kappa$ B—I $\kappa$ B $\alpha$  or I $\kappa$ B $\beta$  (4, 5). The cloning of I $\kappa$ B $\alpha$  (16, 17) allowed progress to be made at several levels. First, it was established that I $\kappa$ B $\alpha$  contains ankyrin repeats (Figure 1). Second, I $\kappa$ B $\alpha$  retains NF- $\kappa$ B in the cytoplasm through masking of the nuclear localization sequences (4, 5, 9). Third, the identification of I $\kappa$ B as an ankyrin repeat containing protein allowed for the prediction that the function of the homologous ankyrin repeats in the precursors of the p50 subunit (NF- $\kappa$ B1) and of the p52 subunit (NF- $\kappa$ B2) was as an intramolecular I $\kappa$ B (see 4, 5). Thus precursors dimerize with another member of the Rel family through the RHD, and the C-terminal ankyrin repeat region functions to retain this dimer in the cytoplasm (also see discussion below). Finally, I $\kappa$ B $\alpha$  exhibited homology with a protein encoded by the gene *bcl-3* found to be translocated in certain lymphomas (see 4, 5, and below).

Immunoprecipitation studies indicate that I $\kappa$ B $\alpha$  is associated predominantly with c-Rel- and RelA-containing dimers. Studies indicate that certain non-Rel

or RelA-containing dimers are likely not to be strongly regulated by I $\kappa$ B. For example, I $\kappa$ B $\alpha$  exhibits a lower affinity for RelB-p52 heterodimers than for RelB-p50 dimers (18), and this likely contributes to constitutive nuclear levels of the former factor. RelB complexes in lymphoid cells may have a lower affinity for I $\kappa$ B $\alpha$  due either to a modification to RelB or to a cell-specific cofactor (19). I $\kappa$ B interaction with NF- $\kappa$ B subunits occurs with residues in the Rel homology domain, and presumed contacts in or around the nuclear localization sequence (NLS) appear to play critical functional roles in inhibiting nuclear localization of NF- $\kappa$ B (4, 9). In addition, data indicate that a single I $\kappa$ B targets the NF- $\kappa$ B dimer (20, 21).

I $\kappa$ B $\alpha$  can be divided into three structural domains: a 70-amino-acid N-terminal region, a 205-amino-acid internal region that is composed of ankyrin repeats, and a C-terminal 42-amino-acid region that contains a so-called PEST region. Mutation and protease sensitivity studies indicate that deletion of the N-terminal or C-terminal region does not inhibit the ability of I $\kappa$ B $\alpha$  to interact with NF- $\kappa$ B (20, 22). However, deletion of the C-terminus does block the ability of I $\kappa$ B $\alpha$  to inhibit DNA binding of NF- $\kappa$ B (20). Mutations within the ankyrin repeat block interactions with NF- $\kappa$ B (4, 5). The discussion below describes important regulatory aspects of the N- and C-terminal regions of I $\kappa$ B $\alpha$ .

Other forms of I $\kappa$ B include the precursors NF- $\kappa$ B1 and NF- $\kappa$ B2, I $\kappa$ B $\gamma$  (an independent protein derived from a unique transcript from NF- $\kappa$ B1) which appears to be limited only to mouse B cells, and Bcl-3 (Figure 1; see 4, 5). The recently cloned I $\kappa$ B $\beta$  is discussed below. Precursor proteins can dimerize with other NF- $\kappa$ B subunits to form dimer molecules that cannot bind to DNA and that cannot translocate into the nucleus (4, 5). NF- $\kappa$ B2 precursor p100 or its C-terminus can form a trimeric complex with a dimer of NF- $\kappa$ B subunits (23, 24), suggesting a different mechanism whereby precursors function in an I $\kappa$ B-like role. Bcl-3 is nuclear in its localization and functions as a transcriptional activator with the p50 or p52 homodimer. Thus the ability of Bcl-3 to interact with these forms of NF- $\kappa$ B results in transcriptional activation rather than an inhibition of nuclear transport or an inhibition of DNA binding (4, 5). Consistent with these results is the observation (25) that the NLS region of p50 is apparently not contacted by Bcl-3, in contrast to the interactions between I $\kappa$ B $\alpha$  and NF- $\kappa$ B subunits.

### *2.3 I $\kappa$ B $\beta$ Appears To Have Properties Distinct from I $\kappa$ B $\alpha$*

As stated above, purification of NF- $\kappa$ B revealed that two forms of I $\kappa$ B were associated with NF- $\kappa$ B dimers. One form, I $\kappa$ B $\alpha$ , is described above and has been intensively studied. Recently, the 46-kDa I $\kappa$ B $\beta$  was purified to homogeneity, and a cDNA clone was derived (26). Like I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  contains ankyrin repeats (Figure 1) and is associated with NF- $\kappa$ B forms in the cytoplasm of various

cells. Like  $I\kappa B\alpha$ ,  $I\kappa B\beta$  preferentially interacts with dimers that contain c-Rel or RelA. mRNA for  $I\kappa B\beta$  is widely expressed, with an especially high level in the testis. Where  $I\kappa B\alpha$  is targeted by a signaling pathway initiated by TNF, IL-1, LPS, and PMA,  $I\kappa B\beta$  reportedly is targeted only by pathways initiated by LPS or by IL-1 (at least in the 70Z/3 pre-B cell line and in the Jurkat T cell line). Further discussion of  $I\kappa B\beta$  follows in the section on signaling and activation of NF- $\kappa$ B.

## 2.4 *I $\kappa$ B-R and I $\kappa$ BL: Functional I $\kappa$ B Molecules?*

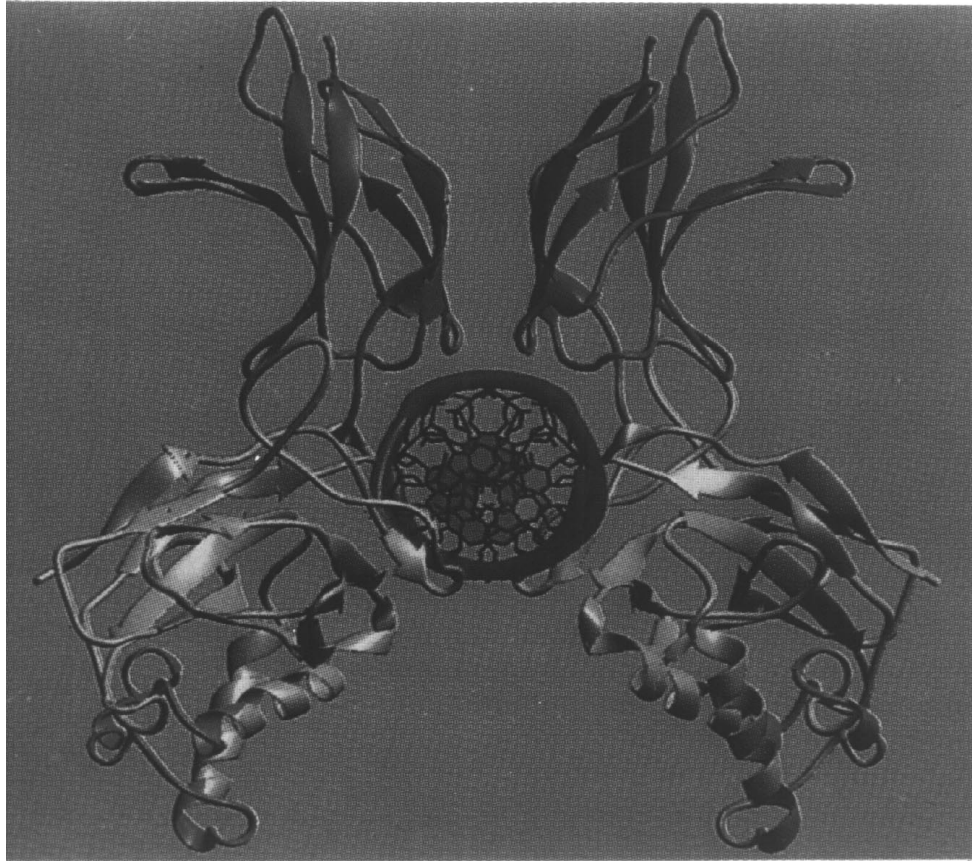
A cDNA clone for an ankyrin repeat-containing protein with homology to mammalian and invertebrate  $I\kappa$ B proteins was recently reported (27). This protein,  $I\kappa$ B-R, is most similar to the *Drosophila*  $I\kappa$ B homolog Cactus (Figure 1 and see below) and is expressed in epithelial cell lines but not in fibroblasts. Two forms of RNA are detected (the smaller RNA is not large enough to encode the described protein), with the larger form expressed in heart and skeletal muscle but not in brain, lung, liver, or kidney. The  $I\kappa$ B-R protein inhibits the DNA binding activity of the p50/RelA dimer and the p50 homodimer but not that of the RelA homodimer, suggesting a preferential interaction with the 50-kDa NF- $\kappa$ B1 subunit. A regulatory role for  $I\kappa$ B-R has not yet been identified.

Another cDNA clone encoding a protein ( $I\kappa$ BL) with homology to  $I\kappa$ B family members has been reported (28). The protein contains two complete and one partial ankyrin repeat and is encoded by a gene in the major histocompatibility complex. No functional studies on this protein have been presented.

## 3. STRUCTURAL AND EVOLUTIONARY STUDIES ON NF- $\kappa$ B

### 3.1 *Crystallography of NF $\kappa$ B1 Bound to DNA*

Two groups recently described the crystal structure of the 50-kDa NF- $\kappa$ B1 homodimer bound to a  $\kappa$ B site (29, 30). In contrast with the relatively small DNA binding domains of many other transcription factors, the DNA binding domain of NF- $\kappa$ B1 encompasses large regions of the RHD. These studies reveal that the RHD folds into two distinct domains similar to those in the immunoglobulin superfamily. DNA contacts are made on the  $\kappa$ B site by both domains, and the C-terminal regions of the RHD constitute the dimerization interface. The overall structure is striking and butterfly-like (see Figure 2). The general relatedness of the RHD in each of the immediate NF- $\kappa$ B family members strongly suggests that a similar overall structure will constitute the DNA binding and dimerizations domains of each family member. However, distinct sequence elements within these domains likely determine different functional properties



*Figure 2* Structure of the Rel homology domain of the p50 NF- $\kappa$ B1 homodimer and its target DNA sequence (see 29, 30). The more N-terminal region of the RHD is shown toward the bottom, and the more C-terminal dimerization region at the top. DNA contacts are made in both domains. (Photograph from Dr. S. Harrison.)

of the different subunits (31). Consistent with previous deletion studies, it is proposed that amino acids in and around the NLS will constitute a composite surface for interaction with I $\kappa$ B. Another important feature is that the minor groove is exposed in the center of the  $\kappa$ B site, allowing for the interaction of HMG-I(Y) with certain  $\kappa$ B sites [see discussion of HMG-I(Y) below].

### 3.2 *NF- $\kappa$ B/Rel Proteins Are Members of a Larger Family*

The factor NF-AT is strongly implicated in the inducible transcription of the IL-2 gene and other cytokine genes. Interestingly, many similarities exist between the NF- $\kappa$ B and the NF-AT family of proteins (see 32 and references therein). Both groups are inducible by activation of T cells, utilizing cytoplasmic/nuclear translocation as a control mechanism, and this activation is blocked by the important immunosuppressant cyclosporin A. Additionally, both NF-AT and NF- $\kappa$ B interact with members of the Fos/Jun family of proteins (32). Furthermore, the core sequence of the NF-AT recognition site, GGAAAA, is very similar to an NF- $\kappa$ B half-site. The recent cloning of members of the NF-AT family demonstrates the existence of a region of NF-AT similar to the Rel homology domain of NF- $\kappa$ B (32; 33). Overall these observations indicate the existence of an extended family of Rel-like proteins that serve critical roles in immune function.

An impressive structural and functional homology exists between the mammalian NF- $\kappa$ B/Rel proteins and two proteins, Dorsal and Dif (see Figure 1), from *Drosophila*. Dorsal is involved in critical developmental pathways determining the dorsal-ventral patterning of the embryo (see 34). Dif serves a protective function, regulating bacteriocidal genes in response to endotoxin (35). These proteins are regulated by an ankyrin repeat containing protein known as Cactus (Figure 1; 34, 36), which functions very similarly to mammalian I $\kappa$ B in controlling nuclear/cytoplasmic localization of Dorsal (37).

## 4. BIOLOGY OF NF- $\kappa$ B

### 4.1 *Expression and Nuclear/Cytoplasmic Status of NF- $\kappa$ B Forms*

Although NF- $\kappa$ B is generally considered to be cytoplasmic in most cell types until stimulation with an inducer, growing evidence provides a more complex view. First, many cell types appear to have moderate levels of the p50 (NF- $\kappa$ B1) homodimer. A role for this factor in constitutive-type transcription is unclear, but it may provide low levels of transcriptional activity or it may serve as a transcriptional repressor (4, 5). Since the p50 dimer can be targeted by Bcl-3, it may serve as a DNA-binding anchor for transcriptional activation by this I $\kappa$ B-related protein (5). A protein similar to or identical to c-Rel is



nuclear during the S phase of the cell cycle (38). Evidence has been provided that NF- $\kappa$ B (other than the p50 homodimer) is nuclear in corneal keratinocytes (39) and in vascular smooth muscles cells (40). Following is a discussion of some other cells where constitutive activation of forms of NF- $\kappa$ B may play important functional roles.

**4.1.1 B CELLS** The one widely accepted example of constitutively active (i.e. nuclear) NF- $\kappa$ B is in B cells. B lymphocytes develop by regulating an ordered expression of immunoglobulin genes, and one role for NF- $\kappa$ B in B cells is in regulating the Ig $\kappa$  gene (7). Pre-B cell lines do not exhibit active NF- $\kappa$ B unless stimulated with cytokines or LPS. However, pre-B cells expanded from Whitlock-Witte bone marrow cultures by treatment with IL-7, and then maintained with a stromal cell feeder layer, exhibited active NF- $\kappa$ B and Ig $\kappa$  transcription (41). Recently several groups have shown that the constitutive form of NF- $\kappa$ B in mature B cell lines is largely the p50-c-Rel heterodimer (42, 43). The p50-RelA heterodimer is found at expected levels in the cytoplasm but is not nuclear in these cells. The mechanism accounting for nuclear levels of c-Rel-p50 is not fully clear but may be explained by significantly increased instability of I $\kappa$ B $\alpha$  and by increased transcription of the c-Rel gene (43, 44).

The functional significance of the constitutive nuclear activation of the c-Rel-p50 heterodimer, and the relative lack of p50-RelA, in mature B cell lines is not clear. Both heterodimers appear to activate Ig $\kappa$  gene expression effectively (43). NF- $\kappa$ B RelA is a potent activator of *c-myc* transcription, as compared to c-Rel, and anti-IgM treatment of a B cell line resulted in increased forms of NF- $\kappa$ B in the nucleus, increased transcription of *c-myc*, and apoptosis (44). Thus, activation of the p50-RelA heterodimer may be deleterious to normal B cell function, resulting in upregulation of *c-myc* and apoptosis. Additional evidence for an important role for NF- $\kappa$ B in B cell biology comes from studies on the B cell surface receptor CD40, which is a member of the TNF receptor family. Engagement of the CD40 receptor activates NF- $\kappa$ B in B cell lines (45, 46), leading to the transcription of a gene encoding a zinc finger protein, A20, which inhibits apoptosis (45). CD40 signaling blocks spontaneous apoptosis of B cells in germinal centers and may be important in selecting B cells undergoing somatic mutation (see 46). The studies with anti-IgM utilized WEH1 231 cells, while the studies with CD40 utilized Louckes and BJAB cells. Thus, NF- $\kappa$ B may have both positive and negative effects on apoptosis in B cells. Additionally, activation of NF- $\kappa$ B by engagement of CD40 is likely to play other important roles in B cells.

**4.1.2 THYMOCYTES** Experiments utilizing freshly isolated thymocytes found that several factors that bind to a  $\kappa$ B site are constitutively activated in these cells

(47). These factors include p50 homodimers, the p50-RelA heterodimer, and c-Rel. Disruption of the thymic microenvironment led to the loss of these binding activities, which suggests that an interaction between lymphocytes and stromal cells provides a stimulus for the activation of NF- $\kappa$ B/Rel nuclear proteins. Some evidence suggests that c-Rel plays a role in the transition from CD4<sup>+</sup>/CD8<sup>+</sup> double positive cells to CD4<sup>+</sup> or CD8<sup>+</sup> single positive cells. Gene knockout studies with p50 or c-Rel do not indicate a role for these subunits in T cell development (see below) but do not rule out a role for RelA.

**4.1.3 NEURONS** Numerous reports analyze the status of  $\kappa$ B binding activities and NF- $\kappa$ B-like forms in the brain. A brain  $\kappa$ B binding protein, called BETA, has been described (48). The BETA complex can be supershifted with antibodies against a large zinc finger protein called MBP-2/AGIE BP-1 (49). Other work has identified two  $\kappa$ B binding complexes, DBF 1 and 2, in the developing rat brain (50). Cross-linking identified the molecular weights of the factors as approximately 110 and 115 kDa. However, these factors are not related to the NF- $\kappa$ B1 and or NF- $\kappa$ B2 factors. Immunohistochemical evidence (51) suggests that both NF- $\kappa$ B p50 and RelA are constitutively active in some neurons of the embryonic rat. This latter work also identified nuclear NF- $\kappa$ B by gel mobility shift assay. Furthermore,  $\kappa$ B-dependent gene expression could be detected in neuronal cell cultures. Experimentation has revealed that vascular adhesion molecule-1 (VCAM-1) is expressed in the developing central nervous system on neuroepithelial cells (52), which are precursors of glial cells and neurons. Utilization of P19 embryonic carcinoma cells as a model of neural differentiation revealed that expression of VCAM-1 correlated with induction of nuclear p50-RelA dimers. NF- $\kappa$ B activity in the brain may be involved with expression of HIV in the central nervous system and may participate in normal brain function.

## 4.2 *Transcriptional Regulation by NF- $\kappa$ B Subunits*

Consistent with their roles as transcription factors, c-Rel, RelB, and RelA contain transcriptional activation domains (53, 54, and see 55). Additionally, both c-Rel and RelA interact with the TATA-binding protein (TBP), and the C-terminus of RelA interacts with the basal factor TFIIB (56 and references therein). Studies in vivo and in vitro indicate that different NF- $\kappa$ B dimers have different transcriptional activation properties (57, 58). Strong evidence indicates that interactions between NF- $\kappa$ B and other transcription factors influence the ability of NF- $\kappa$ B to regulate gene expression in a selective manner. As described below, interactions between NF- $\kappa$ B proteins, HMG-I(Y), IRF-1, and bZIP proteins regulate inducible expression of the interferon  $\beta$  gene. Interactions between NF- $\kappa$ B proteins and bZIP proteins are implicated in the inducible regulation of the genes encoding IL-8, E-selectin, and G-CSF,

for example (see below). A complex that binds to enhancer A in the class I MHC promoter and that is modulated by interferon  $\gamma$  or glucocorticoids reportedly contains the p50 NF- $\kappa$ B subunit in association with the bZIP protein fra-2 (59). Additionally, a DNA-binding complex containing C/EBP and NF- $\kappa$ B proteins has been identified in avian lymphoid cells (60). An interaction between the NF- $\kappa$ B subunit RelA and the zinc finger protein Sp1 has been identified and appears to regulate transcription directed by the HIV-1 LTR (61). The functional outcome of transcriptional induction based on some of these interactions likely involves cooperative DNA binding as well as transcriptional synergy.

### 4.3 *Genes Regulated by NF- $\kappa$ B*

Extensive research has established a clear role for NF- $\kappa$ B in the inducible regulation of a wide variety of genes involved in immune function and inflammation responses (for example, GM-CSF, IL-6, IL-8, IL-2, IL-2R $\alpha$ , etc). An extensive list of genes regulated by NF- $\kappa$ B is provided in previous reviews (4, 5). Following is a discussion of several of these.

The group of genes encoding cell adhesion molecules has been studied extensively for an involvement of NF- $\kappa$ B in their regulation. Vascular cell adhesion molecule-1 (VCAM-1) is a cell surface protein typically found on endothelial cells following exposure to TNF, IL-1, or LPS. VCAM-1 is a member of the immunoglobulin superfamily and binds circulating monocytes and lymphocytes expressing  $\alpha$ 4 $\beta$ 1 or  $\alpha$ 4 $\beta$ 7 integrins and likely participates in the recruitment of these cells to sites of tissue injury. VCAM-1 is also implicated in other cellular processes because it is expressed in the developing central nervous system, in human lymph nodes, and on bone marrow stromal cells. The promoter of the VCAM-1 gene contains two essential NF- $\kappa$ B sites (62), which are not sufficient for activation, and may require an IRF-1 site that is located 3' to the TATA box (63). Interestingly, IRF-1 was found to be inducible by cytokine treatment. Furthermore, IRF-1 physically interacts with NF- $\kappa$ B subunits, and its binding is stimulated by binding of NF- $\kappa$ B and HMG-I(Y). The functional requirement for IRF-1 may explain the cell-type specificity of the VCAM-1 promoter. IRF-1 and NF- $\kappa$ B may be important in the cytokine-induced regulation of multiple promoters. Other genes encoding cell adhesion molecules, such as E-selectin, ICAM-1, and MAd-CAM-1, are also regulated by NF- $\kappa$ B (64–67). Expression of E-selectin requires NF- $\kappa$ B, the bZIP protein ATF-2, and HMG-I(Y) (64, 65). Additionally, interactions between HMG-I(Y), NF- $\kappa$ B, and an ets-like protein (Elf-1) reportedly regulate expression of the IL-2 receptor  $\alpha$  gene (68).

Regulation of the interferon  $\beta$  gene has been intensively studied by Maniatis and colleagues (69, 70). The human gene is induced by viral infection of cells or treatment with double-stranded RNA. One of the regulatory sites (GGGAAA-TTCC; PRDII) in the IFN $\beta$  promoter interacts with both NF- $\kappa$ B and the high

mobility group protein HMG I(Y). HMG I(Y) stimulates the binding of NF- $\kappa$ B to PRDII by binding to the A/T-rich core sequence. Mutations that block either NF- $\kappa$ B or HMG I(Y) binding significantly block activation. These workers propose a model in which interactions between bZip proteins (ATF-2 and c-Jun), IRF-1, HMG I(Y), and NF- $\kappa$ B form a higher order complex, which is required for full activation of the IFN $\beta$  promoter (70). Thus a precise arrangement of binding sites and a  $\kappa$ B site that interacts with HMG-I(Y) are required for the proper transcriptional induction of the IFN $\beta$  gene. Strong similarities exist between the inducible regulation of the IFN $\beta$  gene and those encoding adhesion molecules (64).

Other genes regulated by NF- $\kappa$ B that encode proteins playing important roles in immune response and inflammation are the genes encoding the peptide transporter TAP1 and the proteasome subunit LMP2 (71) as well as the MHC class II invariant chain gene (72). Additionally, genes encoding tissue factor (15, 73), inducible nitric oxide synthase (iNOS), and various cytokines are regulated by NF $\kappa$ B. iNOS catalyzes the high output of NO, and the iNOS gene is transcriptionally activated in response to LPS and interferon gamma. The inducible activation of NF- $\kappa$ B stimulates the transcription of the iNOS gene (74), leading to an increase in NO production. As discussed below, the production of NO may feed back to inhibit NF- $\kappa$ B activation. NF- $\kappa$ B is strongly implicated in the transcriptional regulation of several cytokine and growth factor genes, including IL-2, IL-6, IL-8, and G-CSF. IL-2 contains an NF- $\kappa$ B site that has been studied by several groups (7). Activators of T cells such as PMA and ionomycin activate NF- $\kappa$ B and its binding to this site. The immunosuppressant cyclosporin A blocks the activation of NF- $\kappa$ B in response to T cell receptor-mediated signals (75). Signaling through CD28, a costimulatory receptor, also leads to the activation and binding of NF- $\kappa$ B/Rel forms to a recently described CD28 response element (CD28RE) in the IL-2 promoter (76). The CD28RE is found upstream of other genes such as IL-3. Furthermore, expression of the IL-8 gene is regulated by NF- $\kappa$ B (77) and uses the CD28 costimulatory pathway in T cells (78). G-CSF is a growth factor for hematopoietic cells produced by mesenchymal and myeloid cells in response to activation by inflammatory stimuli. A cooperative interaction between C/EBP $\beta$  (NF-IL6) and the RelA subunit of NF- $\kappa$ B is important in the regulation of G-CSF in response to TNF treatment (79).

Although NF- $\kappa$ B/Rel proteins are strongly implicated in the regulation of genes involved in the immune system and in inflammation, these transcription factors also regulate genes involved in control of cell growth. Two NF- $\kappa$ B sites have been identified in the *c-myc* promoter/enhancer region (80). The induction of *c-myc* gene expression either by IL-1 or by the HTLV-I tax protein functions

through the two NF- $\kappa$ B sites. As discussed previously, classical NF- $\kappa$ B or p65 homodimers appear to be the best activators of *c-myc* as Rel is only a weak activator. NF- $\kappa$ B binding proteins are also implicated in expression of the translocated *c-myc* allele in Burkitt's lymphoma (81) and in the transcriptional regulation of the p53 gene (82).

#### 4.4 *Roles for NF- $\kappa$ B Subunits Revealed in Gene Knockout Studies*

Knockout of most of the genes encoding NF- $\kappa$ B/Rel subunits and two of the I $\kappa$ B forms has been recently accomplished. The studies confirm the importance of NF- $\kappa$ B/Rel proteins in immune function and have revealed several unexpected findings.

4.4.1 NF- $\kappa$ B1 Mice lacking the p50/p105 (NF- $\kappa$ B1) subunits develop normally but exhibit defects in immune responses involving B cells (83). In these mice, the ability of B cells to proliferate in response to LPS is defective, and the production of antibodies is impaired. Total serum Ig was approximately four-fold lower in the knockout mice, and IgE was reduced approximately 40-fold. This suggests that p50 plays an important role in heavy-chain class switching. Interestingly, p50 is reportedly (84) a critical factor for an IL-4-responsive region in the Ig heavy-chain germline  $\epsilon$  promoter, responsible for switching to IgE. NF- $\kappa$ B1  $-/-$  mice cannot effectively clear the pathogens *Listeria* or *Streptococcus* and exhibit increased resistance to infection by EMC virus (83). This resistance was correlated with increased expression of IFN $\beta$ , an important antiviral protein. Thus p50 homodimers likely negatively regulate expression of the IFN $\beta$  gene.

4.4.2 RELB Like the NF- $\kappa$ B1 knockout mice, the RelB knockout mice do not show a developmental phenotype but do show defects in normal immune function and in hematopoiesis (85, 86). It had previously been speculated that RelB plays an important role in the constitutive type expression of  $\kappa$ B-regulated genes in lymphoid cells. RelB is expressed in the spleen, thymus, lymph nodes, and intestine. In the thymus, RelB transcripts are concentrated in the medulla with high levels of RelB in the nucleus of interdigitating dendritic cells. The RelB  $-/-$  mice exhibited multifocal and mixed infiltration of inflammatory cells in several tissues. Additionally, the mice exhibited splenomegaly, reduced antigen-presenting dendritic cells in the thymus, myeloid hyperplasia, and impaired cellular immunity. These studies demonstrate an important role for RelB in immune function and in the differentiation of dendritic cells and thymic medullary epithelial cells. Furthermore, the data show that the loss of RelB cannot be complemented by another member of the NF- $\kappa$ B/Rel

family and, therefore, that there is no simple redundancy within the NF- $\kappa$ B/Rel proteins. It will be important to identify the relevant genes regulated by RelB that contribute to these immunological functions.

**4.4.3 RELA** The RelA (p65) null mice exhibit a dramatic phenotype—embryonic lethality apparently due to widespread apoptosis within the liver (87). Day-14 embryos exhibit a normal liver phenotype, but by day 16 the liver (specifically hepatocytes) has undergone extensive apoptosis. This phenotype is similar to that observed with the c-Jun knockout and suggests that NF- $\kappa$ B and Jun may serve similar functions in development of the liver (see 87). The normal induction of the p50-RelA heterodimer by TNF was lost in the RelA null mice as was the inducibility of NF- $\kappa$ B-regulated genes such as *I $\kappa$ B $\alpha$*  and *GM-CSF*.

**4.4.4 c-REL** The c-Rel knockout mouse develops normally, and cells from all hemopoietic lineages are normal; however, mature B and T cells are not responsive to certain mitogenic stimuli (88). The c-Rel  $-/-$  animals exhibit normal Ig $\kappa$  levels on splenic B cells and normal IL-2R $\alpha$  chain on thymic T cells; thus, c-Rel is not required for expression of these genes containing  $\kappa$ B binding sites. Proliferation of B cells induced by LPS, CD40, or anti-IgM was defective for unknown reasons. A defective T cell—proliferative response induced by engagement of the T cell receptor was observed, and CD28 costimulation did not overcome this defect. Interestingly, proliferation of cells induced by PMA and ionomycin was near normal. The inability of c-Rel  $-/-$  T cells to respond to anti-TCR stimuli and CD28 costimulation was correlated with loss of production of IL-2. Indeed, this proliferative block could be overcome by the addition of exogenous IL-2. Thus, it appears that c-Rel is required for the CD28 responsive element to be functional (see discussion above). Additionally, the c-Rel deficient mice displayed deficient immunoglobulin production in unchallenged animals, and T cell—dependent humoral immune responses to antigenic challenge were also impaired.

**4.4.5 I $\kappa$ B $\alpha$  AND BCL-3** *I $\kappa$ B $\alpha$*  null mice are apparently normal at birth but then enter a wasting phase and die approximately 7 days after birth (89). The runt mice have small spleens and thymuses, enhanced granulopoiesis, and scaly skin. NF- $\kappa$ B is constitutively activated in splenocytes and thymocytes as well as in other cells. Upregulation of some genes expected to be regulated by NF- $\kappa$ B (G-CSF and VCAM-1) is detected. The loss of *I $\kappa$ B $\alpha$*  leads to the nuclear localization of NF- $\kappa$ B in most cell types, most prominently in the spleen and thymus. This latter result suggests that *I $\kappa$ B $\alpha$*  is the dominant *I $\kappa$ B* in these organs. Treatment of *I $\kappa$ B $\alpha$*   $-/-$  embryonic fibroblasts with TNF leads to activation of NF- $\kappa$ B through targeted degradation of *I $\kappa$ B $\beta$* , showing that at least

in some cell types this inhibitor can be targeted by TNF. Furthermore, nuclear NF- $\kappa$ B levels remained elevated longer in  $-/-$  animals following treatment with TNF, demonstrating a role for I $\kappa$ B $\alpha$  in controlling the postinduction repression of NF- $\kappa$ B. Clearly, the I $\kappa$ B $\beta$  knockout studies will be informative in understanding potential differential roles for the two major I $\kappa$ B forms. Evidence for a role in constitutive activation of NF- $\kappa$ B in the phenotype of the I $\kappa$ B $\alpha$   $-/-$  mice is the observation that a cross between these mice and the p50  $-/-$  mice extends the life expectancy of the former (89).

The Bcl-3 gene knockout has been derived very recently. So far, no obvious developmental or immunological abnormalities have appeared in the mice (A Berns, I Verma, personal communication).

## 5. MECHANISMS FOR THE ACTIVATION AND CONTROL OF NF- $\kappa$ B ACTIVITY

Enormous progress has been made toward identifying the mechanisms whereby NF- $\kappa$ B is activated to move into the nucleus in response to numerous stimuli. Specific molecular events involved in this activation are now well described and offer insights into a fascinating control mechanism. Despite this progress, many important questions remain regarding the pathways and mechanisms of NF- $\kappa$ B activation.

### 5.1 *Phosphorylation of I $\kappa$ B $\alpha$ Is Coupled To Its Proteolysis via the Ubiquitin Pathway*

In vivo analysis has shown that inducer-mediated activation of NF- $\kappa$ B is correlated with the hyperphosphorylation of I $\kappa$ B $\alpha$  and its subsequent degradation (reviewed in 4–12). Paralleling the loss of I $\kappa$ B $\alpha$  in the cytoplasm is the appearance of NF- $\kappa$ B in the nucleus. Reagents such as antioxidants or certain protease inhibitors that inhibit phosphorylation block the appearance of nuclear NF- $\kappa$ B. Based on studies showing that I $\kappa$ B $\alpha$  is intrinsically unstable when not associated with NF- $\kappa$ B, phosphorylation of I $\kappa$ B $\alpha$  was generally thought to lead to its dissociation from NF- $\kappa$ B, which resulted in proteolysis (9). Recent experiments have offered a different interpretation.

One set of experiments (90–96) showed that hyperphosphorylated I $\kappa$ B $\alpha$  could be coimmunoprecipitated with NF- $\kappa$ B, suggesting that inducible phosphorylation does not cause the dissociation of I $\kappa$ B $\alpha$  from NF- $\kappa$ B. Second, experiments with a class of protease inhibitors called peptide aldehydes demonstrated that these compounds block the degradation of I $\kappa$ B $\alpha$  and the nuclear appearance of NF- $\kappa$ B, but do not block inducible phosphorylation of I $\kappa$ B $\alpha$  (90–97). In addition, these studies led to the important proposal that a target of peptide aldehydes, the proteasome, is responsible for the signal-mediated

degradation of  $\text{I}\kappa\text{B}\alpha$  (97). Like  $\text{I}\kappa\text{B}\alpha$ ,  $\text{I}\kappa\text{B}\beta$  is targeted for degradation in response to the inducers LPS and IL-1. Evidence that  $\text{I}\kappa\text{B}\beta$  is degraded by the proteasome is provided by data that show that peptide aldehydes specific for the proteasome block its degradation (T Finco, J Reuther, A Beg, D Ballard, S Shenolikar, A Baldwin, submitted).

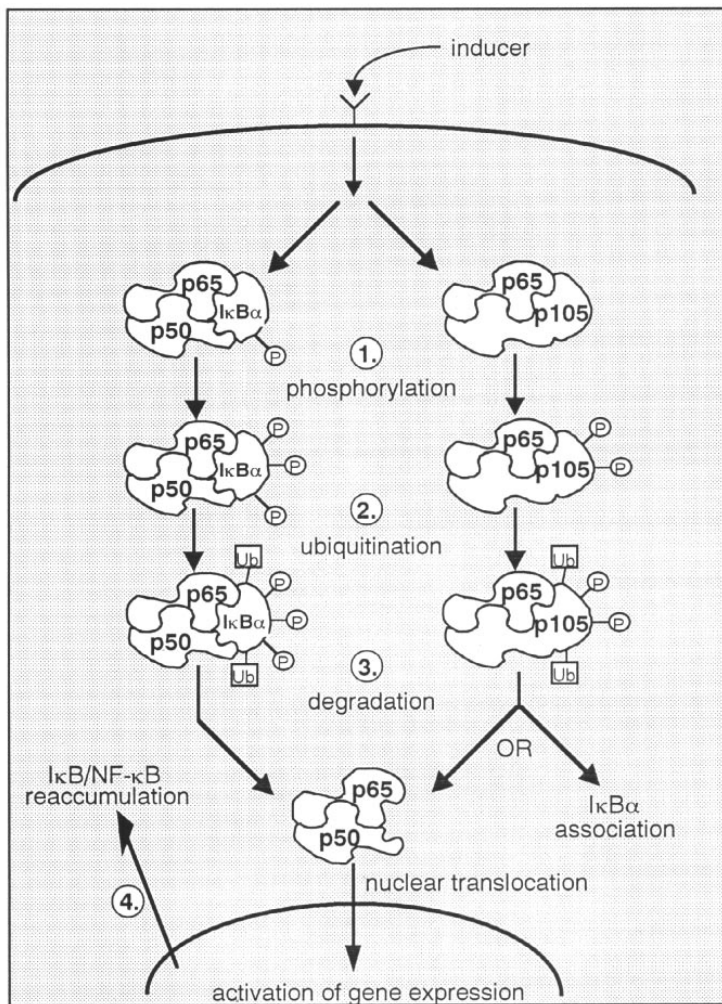
What mechanism targets  $\text{I}\kappa\text{B}\alpha$ , and presumably  $\text{I}\kappa\text{B}\beta$ , for degradation? Evidence that phosphorylation precedes degradation (95) suggests that phosphorylation of  $\text{I}\kappa\text{B}\alpha$  is required for degradation. Mutation of two serines near the N-terminus of  $\text{I}\kappa\text{B}\alpha$ , ser32 and ser36, blocked inducible phosphorylation and also blocked degradation (98–100). Phosphopeptide mapping demonstrates that these two serines are inducibly phosphorylated in response to various activators of NF- $\kappa\text{B}$  (J DiDonato, M Karin, personal communication). Recent work indicates that  $\text{I}\kappa\text{B}\alpha$  is ubiquitinated in response to inducer, and mutation of ser32 and ser36 inhibits the inducible ubiquitination (101). Importantly, ubiquitinated  $\text{I}\kappa\text{B}\alpha$  is degraded by purified preparations of proteasomes. Mutation of two lysine residues in the N-terminus block ubiquitination and degradation of  $\text{I}\kappa\text{B}\alpha$  but not induced phosphorylation (102). Presumably these two lysines are the targets for ubiquitination. Thus the current model is that targeted phosphorylation of  $\text{I}\kappa\text{B}$  leads to the ubiquitination of this protein, which leads to degradation by the proteasome (see Figure 3). A question raised by these data is, what makes the phosphorylated form of  $\text{I}\kappa\text{B}$  a substrate for the ubiquitination pathway? Possibly the lysine residues to be ubiquitinated are masked by secondary or tertiary structure until the nearby serines are phosphorylated.

Although inducible phosphorylation of  $\text{I}\kappa\text{B}\alpha$  (and presumably  $\text{I}\kappa\text{B}\beta$ ) plays a critical role in the activation of NF- $\kappa\text{B}$ ,  $\text{I}\kappa\text{B}\alpha$  is also constitutively phosphorylated. Recent evidence suggests that casein kinase II (CKII) can associate with and phosphorylate the C-terminal region of  $\text{I}\kappa\text{B}\alpha$  (103; J Hiscott, personal communication). Interestingly, deletion of the C-terminus inhibits degradation (but not association with NF- $\kappa\text{B}$ ) (98, 104, 105) and also abrogates the ability of  $\text{I}\kappa\text{B}\alpha$  to inhibit DNA binding by NF- $\kappa\text{B}$  (20). Thus, constitutive phosphorylation of  $\text{I}\kappa\text{B}\alpha$  in the C-terminal region may be required for degradation, possibly mediated by the PEST residues found in this domain. Additional roles for constitutive phosphorylation of  $\text{I}\kappa\text{B}\alpha$  may include regulation of the stability of free  $\text{I}\kappa\text{B}$  or assembly with NF- $\kappa\text{B}$  subunits.

## 5.2 *NF- $\kappa\text{B}$ Subunits Are Targeted for Inducible Phosphorylation*

Both the NF- $\kappa\text{B}$ 1 (p105) and NF- $\kappa\text{B}$ 2 (p100) proteins are cytoplasmic and are associated with different NF- $\kappa\text{B}$ /Rel proteins. As discussed above, this association may be as dimers or, as recently proposed, trimers. Several of the NF- $\kappa\text{B}$





**Figure 3** Generalized NF-κB activation scheme. Following exposure of a cell to inducer, IκB (either IκBα, IκBβ or one of the precursor proteins) becomes phosphorylated (*step 1*) by a presently unknown kinase(s). (Note that IκBα exhibits a basal level of phosphorylation.) The IκB form then becomes ubiquitinated (*step 2*) and degraded by the proteasome (*step 3*). In the case of p105, the degradation is partial (generating p50). NF-κB then translocates to the nucleus where it activates a variety of genes (*step 4*) including IκBα and p50/p105. See text for details.

inducers lead to the degradation of the p105 precursor protein with the appearance of the p50 form (4, 5). This degradation is limited to the C-terminal half of the protein (97). As with  $\text{I}\kappa\text{B}\alpha$  targeting, degradation appears to be preceded by inducible phosphorylation (106, 107) and is mediated by ubiquitination and processing by the proteasome (see Figure 3; 97). Degradative processing of p105 that is in association with other NF- $\kappa$ B subunits (for example, RelA) may lead to nuclear translocation directly or to association with an  $\text{I}\kappa\text{B}$  form (see Figure 3).

Reagents that induce NF- $\kappa$ B activation via the inducible phosphorylation of  $\text{I}\kappa\text{B}$  reportedly also lead to the inducible phosphorylation of subunits other than those for the precursor/ $\text{I}\kappa\text{B}$ -like forms. For example, the RelA subunit becomes rapidly phosphorylated in response to TNF (108; D Wang, T Finco, A Baldwin, unpublished). A role for this inducible phosphorylation has been suggested to involve enhancement of DNA binding (106) but may also be correlated with release from  $\text{I}\kappa\text{B}$ , nuclear translocation, activation of transcription functions, or for differential targeting of NF- $\kappa$ B forms that may be associated with  $\text{I}\kappa\text{B}\alpha$  or  $\text{I}\kappa\text{B}\beta$  (see below).

### 5.3 *Mutual Regulation of NF- $\kappa$ B and $\text{I}\kappa\text{B}$*

Initial studies that demonstrated the correlation of  $\text{I}\kappa\text{B}\alpha$  loss with the appearance of nuclear NF- $\kappa$ B also indicated that  $\text{I}\kappa\text{B}\alpha$  levels recovered within an hour or so (see 4, 5 for reviews). This reappearance of  $\text{I}\kappa\text{B}\alpha$  depended on protein synthesis and correlated with the inducible expression of  $\text{I}\kappa\text{B}\alpha$  mRNA. Furthermore, transfection into cells of an expression vector for RelA led to an increase in  $\text{I}\kappa\text{B}\alpha$  mRNA. It was therefore proposed that nuclear NF- $\kappa$ B caused the transcriptional activation of the  $\text{I}\kappa\text{B}\alpha$  gene. Promoter analysis has confirmed that the  $\text{I}\kappa\text{B}\alpha$  gene contains multiple NF- $\kappa$ B binding sites and that these sites are functional in the upregulation of gene expression in response to inducers that activate NF- $\kappa$ B (see 4, 5). Evidence has been presented, however, that adherent monocytes (from which  $\text{I}\kappa\text{B}\alpha$  was initially cloned) upregulate  $\text{I}\kappa\text{B}\alpha$  mRNA through a nontranscriptional event (109). These results indicate that NF- $\kappa$ B and  $\text{I}\kappa\text{B}$  are components of a mutual regulatory system in which the levels of one regulatory component control the activity or quantity of the other.

The rapid reaccumulation of  $\text{I}\kappa\text{B}\alpha$  following its loss is apparently important in reestablishing cytoplasmic pools of NF- $\kappa$ B/ $\text{I}\kappa\text{B}$  complexes. Additionally, this reaccumulation appears to repress NF- $\kappa$ B activity following induction because resynthesized  $\text{I}\kappa\text{B}\alpha$  enters the nucleus, interacts with NF- $\kappa$ B forms, and inhibits DNA binding (110). Consistent with this model is the observation that  $\text{I}\kappa\text{B}\alpha$   $-/-$  cells exhibit high nuclear levels of NF- $\kappa$ B for long times following induction with TNF $\alpha$  (89). A mechanism for removal of NF- $\kappa$ B from the

nucleus is suggested by the presence of a potential nuclear export sequence in  $\text{I}\kappa\text{B}\alpha$  (111). Thus  $\text{I}\kappa\text{B}\alpha$ , and not  $\text{I}\kappa\text{B}\beta$  (see below), is considered to be important for the postinduction repression of  $\text{NF-}\kappa\text{B}$ .

## 5.4 *Similarities and Differences Between $\text{I}\kappa\text{B}\alpha$ - and $\text{I}\kappa\text{B}\beta$ -Controlled Pathways*

As discussed previously, many similarities exist between  $\text{I}\kappa\text{B}\alpha$  and  $\text{I}\kappa\text{B}\beta$ . Evidence indicates, however, that  $\text{I}\kappa\text{B}\beta$  does not respond to treatment with TNF or PMA but does respond to LPS or IL-1 in pre-B or Jurkat T cells (26). In addition,  $\text{I}\kappa\text{B}\beta$  reportedly does not reaccumulate following induction of  $\text{NF-}\kappa\text{B}$ ; thus a persistent activation of  $\text{NF-}\kappa\text{B}$  follows. Mechanisms that would prevent  $\text{I}\kappa\text{B}\alpha$ , which rapidly reaccumulates following induction, from inhibiting the  $\text{I}\kappa\text{B}\beta$ -released  $\text{NF-}\kappa\text{B}$  have been proposed. One idea is that  $\text{NF-}\kappa\text{B}$  that is associated with  $\text{I}\kappa\text{B}\beta$  would be modified (possibly by phosphorylation) so that it could not be targeted for inhibition by  $\text{I}\kappa\text{B}\alpha$ . Many issues remain to be clarified regarding differences between  $\text{I}\kappa\text{B}\alpha$  and  $\text{I}\kappa\text{B}\beta$ . One question is whether different kinases function to target the two forms of  $\text{I}\kappa\text{B}$ . Evidence for this is that  $\text{I}\kappa\text{B}\beta$  is targeted for degradation with slower kinetics than  $\text{I}\kappa\text{B}\alpha$  and that there is apparent inducer specificity in the targeting of the two  $\text{I}\kappa\text{B}$  forms. However, recent evidence (J DiDonato, M Karin, personal communication) indicates that serines in the N-terminus of  $\text{I}\kappa\text{B}\beta$ , in homologous positions to ser32 and ser36 in  $\text{I}\kappa\text{B}\alpha$ , play critical roles in the targeted degradation of this protein. This would indicate that a kinase that is identical or very similar to the  $\text{I}\kappa\text{B}\alpha$  kinase would target  $\text{I}\kappa\text{B}\beta$ . Additionally, it is not clear whether targeting of  $\text{I}\kappa\text{B}\beta$  occurs in all cell types (see discussion above relevant to the  $\text{I}\kappa\text{B}\alpha$  gene knockout).

## 5.5 *Tangled Evidence for the Signal Transduction Pathways*

**5.5.1 KINASES AND PHOSPHATASES** Much research is now directed at elucidating the signal transduction pathways involved in controlling activation of  $\text{NF-}\kappa\text{B}$ . It is clear that the many different inducers initiate their pathways through distinct receptors. How these different responses converge on  $\text{I}\kappa\text{B}$  is still unknown. Mutations of ser 32 and 36 inhibit activation of  $\text{NF-}\kappa\text{B}$  controlled by T cell activation signals PMA, TNF, LPS, HTLV-I tax, and okadaic acid. Thus, a single kinase activated by multiple pathways may target these residues in  $\text{NF-}\kappa\text{B}$ .

Nevertheless, several kinases have been implicated in the activation of  $\text{NF-}\kappa\text{B}$ . The best data exist for the double-stranded RNA activated kinase (PKR), which phosphorylates  $\text{I}\kappa\text{B}\alpha$  in vitro (112). Furthermore, in vivo inactivation of this kinase inhibits the ability of double-stranded RNA to activate  $\text{NF-}\kappa\text{B}$  (113). However, this inactivation did not block activation of  $\text{NF-}\kappa\text{B}$  by TNF

and presumably other inducers. It would be interesting to map the potential phosphorylation sites on  $\text{I}\kappa\text{B}\alpha$  targeted by dsRNA-activated kinase. Another kinase, Raf-1, has been proposed, to target  $\text{I}\kappa\text{B}\alpha$  (114). Evidence for an involvement of Raf in the activation of gene expression directed through a  $\kappa\text{B}$  site has been presented through the utilization of constitutively active and dominant negative vectors (115). Activation of Ras or Raf may not lead to the nuclear translocation of  $\text{NF-}\kappa\text{B}$ , but it can activate gene expression through a  $\kappa\text{B}$  site. The mechanism is unclear but may involve the stimulation of the transcriptional activation domains of constitutively nuclear forms of  $\text{NF-}\kappa\text{B}$  (T Finco, A Baldwin, unpublished). Dominant negative experiments as well as direct expression experiments have implicated  $\text{PKC}\zeta$  as a regulator of  $\text{NF-}\kappa\text{B}$  activation (116). Due to their activation by inducers of  $\text{NF-}\kappa\text{B}$  such as inflammatory cytokines, UV, and LPS, there has been speculation that members of the stress-activated protein kinases (SAPKs) or their regulators may play a role in  $\text{I}\kappa\text{B}\alpha$  phosphorylation, but no published data exist regarding this possibility. Additionally, a kinase reportedly associated with  $\text{NF-}\kappa\text{B}/\text{I}\kappa\text{B}$  complexes also appears to phosphorylate the  $\text{NF-}\kappa\text{B}$  subunits (117).

Phosphatases likely play an important role in the activation of  $\text{NF-}\kappa\text{B}$ , either regulating kinase pathways that may control the signal transduction pathway or by directly dephosphorylating  $\text{I}\kappa\text{B}$ . Based on inhibition by cyclosporin A and on transfection studies, the  $\text{Ca}^{2+}$ -dependent phosphatase calcineurin is apparently involved in the activation of  $\text{NF-}\kappa\text{B}$  in T cells (75). In addition, FK506, an inhibitor of calcineurin, blocks the activation of c-Rel in B and T cells (118). The target for calcineurin in this pathway is not known. Inhibitors of the ser/thr phosphatases PP1 and PP2A, such as okadaic acid, potentially activate  $\text{NF-}\kappa\text{B}$ , suggesting the involvement of a phosphatase in regulating some aspect of the pathway. Evidence that PP2A can directly dephosphorylate  $\text{I}\kappa\text{B}\alpha$  has been provided (T Finco, J Reuther, A Beg, D Ballard, S Shenolikar, A Baldwin, submitted; 119).

**5.5.2 CERAMIDE, REACTIVE OXYGEN INTERMEDIATES, AND REDOX STATE** Second messengers may be involved in the early activation pathway of  $\text{NF-}\kappa\text{B}$ . The generation of ceramide in response to TNF or IL-1 may be critical in initiating the events leading to  $\text{NF-}\kappa\text{B}$  activation via degradation of  $\text{I}\kappa\text{B}\alpha$  (see 120 and references therein). According to the current model, the interaction of TNF with the 55-kDa TNF receptor activates an acidic sphingomyelinase (Smase) through the generation of diacylglycerol by a phosphatidylcholine-specific phospholipase C. Smase leads to the production of ceramide. That addition of ceramide or sphingomyelinase to cells leads to the activation of  $\text{NF-}\kappa\text{B}$  (see 120) supports this model. Recent evidence indicates that addition of acidic SMase to a cell-free system leads to the degradation of  $\text{I}\kappa\text{B}\alpha$  (121). Based on these data, it has been

proposed that the generation of ceramide leads to the activation of a kinase that phosphorylates  $I\kappa B\alpha$ . There is also evidence against a role for ceramide in the activation of NF- $\kappa$ B. Cells deficient in acidic SMase can activate NF- $\kappa$ B in response to TNF or IL-1 (122). Possibly another SMase is involved in the process of activation of ceramide in these cells. Second, inhibition of the ceramide pathway by chronic stimulation of cells with PMA does not inhibit the ability of TNF to activate NF- $\kappa$ B (123). Finally, levels of ceramide that activate the Jun kinase (JNK) are not sufficient to activate NF- $\kappa$ B (124). Clearly more experimentation is needed to resolve whether ceramide is an initiator of NF- $\kappa$ B activation, and, if so, what regulatory pathways exist downstream of this molecule.

Reactive oxygen intermediates (ROIs) have also been proposed to be involved in the activation of NF- $\kappa$ B (4). This is based on the observations that treatment of some cells with  $H_2O_2$  can activate NF- $\kappa$ B and that certain antioxidants such as N-acetyl cysteine or PDTC can block activation of NF- $\kappa$ B by blocking the signal-induced phosphorylation of  $I\kappa B\alpha$ . Additionally, many of the known inducers of NF- $\kappa$ B lead to the generation of ROIs. Compelling arguments for the role of ROIs in the activation of NF- $\kappa$ B have been presented by Baeuerle and colleagues (4, 125). These authors propose that the initial response to TNF and other inducers is the production of superoxide anion followed by the generation of  $H_2O_2$  (125). Identification of signaling components that may function downstream of  $H_2O_2$  in the potential activation of NF- $\kappa$ B is lacking. Arguments against ROI involvement in NF- $\kappa$ B activation have been published recently (126, 127).

Activation of NF- $\kappa$ B binding by thioredoxin, a protein involved in maintaining the redox environment of the cell, involves the reduction of a disulfide bond at cys62 of the p50 subunit (see 128). The sequence of this region is conserved in many of the NF- $\kappa$ B/Rel family proteins. Recently, the structure of thioredoxin and a peptide encompassing the cys62 region has been reported (128). This part of p50 is found in the L1 loop and makes apparently critical contacts with the  $\kappa$ B DNA binding site. Expression of thioredoxin increases following exposure of cells to UV light or to  $H_2O_2$  (129), suggesting a possible role in increasing NF- $\kappa$ B activity in response to these inducers.

## 5.6 *Hypoxia Appears To Activate NF- $\kappa$ B via a Unique Pathway*

Exposure of cells to low oxygen concentrations results in the activation of NF- $\kappa$ B (130), which is likely to be important in situations such as angiogenesis associated with tumorigenesis, and in ischemia. Not surprisingly, activation of NF- $\kappa$ B by hypoxia is correlated with the loss of  $I\kappa B\alpha$ . However, an unexpected result was that  $I\kappa B\alpha$  is apparently tyrosine phosphorylated in hypoxic cells (130). This would indicate that a kinase is involved in the phosphorylation of  $I\kappa B\alpha$  that is distinct from that utilized in responses to TNF, IL-1, LPS, or

PMA. Interestingly, it has been proposed that Ras and Raf, but not Map, kinases are involved in this activation (131). These results need to be followed up, the phosphorylation site(s) mapped, and the functional consequence of the reported tyrosine phosphorylation tested.

### 5.7 *Activation of NF- $\kappa$ B by Viral Proteins*

Many viral gene products activate NF- $\kappa$ B. This may be advantageous to the virus for several reasons. First, several viruses (such as HIV, CMV, and SV40) have NF- $\kappa$ B binding sites in their promoter/enhancer regions. Second, NF- $\kappa$ B appears to regulate genes required for viral replication. For example, a factor required for the HIV Rev splicing function may be regulated by NF- $\kappa$ B (132). Finally, activation of NF- $\kappa$ B may provide a favorable environment for viral replication, possibly by serving as a mitogenic regulator.

Probably the most intensively studied viral protein known to activate NF- $\kappa$ B is the Tax protein of HTLV-I. Tax has been proposed to activate NF- $\kappa$ B by different mechanisms. One mechanism appears to be through a direct physical interaction with the NF- $\kappa$ B2 100-kDa protein (24, 133, 134). Second, evidence has been provided that Tax activates a signal transduction pathway leading to phosphorylation and degradation of I $\kappa$ B and to the nuclear appearance of NF- $\kappa$ B (90, 135, 136). Finally, Tax may directly interact with NF- $\kappa$ B subunits bound to their target sites and stimulate transcription (137). Activation of NF- $\kappa$ B by Tax is likely to play an important role in the pathogenesis of HTLV-I, possibly through the upregulation of genes encoding IL-2, IL-2R $\alpha$ , and IL-6 (138). It has been proposed that the HIV Tat protein activates NF- $\kappa$ B (139). Other evidence indicates that Tat potentiates NF- $\kappa$ B activation by TNF by altering the cellular redox state (140). Recent evidence suggests that the  $\kappa$ B sites in the HIV LTR are absolutely required for induction of LTR-reporter gene activity in peripheral CD4<sup>+</sup> T cells in response to Tat (141). Another important HIV protein, nef, can activate NF- $\kappa$ B when functioning as a cell membrane-associated protein or can inhibit its activation if expressed cytoplasmically (142, 143). A transforming protein of Epstein-Barr virus, the latent membrane protein (LMP-1), has been shown to activate NF- $\kappa$ B (144–147). A mechanism for activation may be that LMP-1 associates with factors that are likely involved with signaling directed by the TNF receptor (148).

**5.7.1 CONCLUSION** It is unlikely that significant progress will be made on the NF- $\kappa$ B signaling pathway(s) until research proceeds directly downstream from known receptors and directly upstream from the final phosphorylated substrates (I $\kappa$ B and NF- $\kappa$ B subunits). For example, progress in this regard has been made through the identification of a protein associated with the TNF receptor that functions in the signaling of NF- $\kappa$ B activation (149). This protein,

TRADD, interacts with the intracellular domain of the 55-kDa TNF receptor (TNF receptor 1). Overexpression of TRADD leads both to apoptosis and to the activation of NF- $\kappa$ B. However, the pathways for activation of NF- $\kappa$ B and for apoptosis appear to be distinct since crmA, an inhibitor of the IL-1 $\beta$ -converting enzyme, blocked apoptosis but not the activation of NF- $\kappa$ B. At the other end of the pathway, the kinase(s) involved in the phosphorylation of I $\kappa$ B and of NF- $\kappa$ B subunits must be identified. Such data will permit definitive dissection of the signaling pathways involved in NF- $\kappa$ B activation.

## 5.8 *Inhibitors of NF- $\kappa$ B Activation*

Studies utilizing compounds that inhibit NF- $\kappa$ B activation may provide insight into the activation pathways, may clarify biological situations where inhibition of NF- $\kappa$ B is functionally important, and suggest therapies for diseases related to the acute or chronic activation of NF- $\kappa$ B. An example is tepoxalin, a dual inhibitor of cyclooxygenase and 5-lipoxygenase, which functions to inhibit NF- $\kappa$ B induction by several inducers in multiple cell types (150). Other examples are discussed below.

**5.8.1 IL-10** Interleukin-10 (IL-10) inhibits the transcription of a variety of cytokine genes associated with TH1 T cell responses. Recently, IL-10 was shown to inhibit the induction of NF- $\kappa$ B by LPS in human peripheral blood mononuclear cells (151). These data provide an important link in understanding the inhibitory functions of IL-10 toward certain cytokines. It is intriguing to speculate that the mucosal inflammation with abnormal TH1 T cell responses seen in IL-10-null mice (152) is due in part to an inability to block the function of NF- $\kappa$ B.

**5.8.2 GLUCOCORTICOIDS, SALICYLATES, AND OTHER IMMUNOSUPPRESSANTS** Glucocorticoids are widely used immunosuppressants. For example, prednisone is used to suppress immune responses in organ or bone marrow transplants, and is also used in chronic inflammatory diseases such as arthritis. Glucocorticoids reportedly inhibit NF- $\kappa$ B by two mechanisms. First, several groups report that activated glucocorticoid receptors directly interact with and inhibit activated NF- $\kappa$ B subunits (153–156). A second mechanism involves the transcriptional activation of the I $\kappa$ B $\alpha$  gene in response to treatment with glucocorticoids (157, 158). Glucocorticoids, by upregulating I $\kappa$ B $\alpha$  protein levels, function to block nuclear translocation of NF- $\kappa$ B and DNA-binding. Salicylates (159), which are nonsteroidal anti-inflammatory drugs, inhibit the activation of NF- $\kappa$ B at concentrations used to treat arthritis.

Cyclosporin A (CsA) and rapamycin, both important immunosuppressants that target T cells, inhibit the activation of NF- $\kappa$ B induced in T cells by different

stimuli. CsA blocks the activation of NF- $\kappa$ B in response to the engagement of the T cell receptor (75). One group has reported that rapamycin blocks the induction of NF- $\kappa$ B following CD28 costimulation in T cells (160). Interestingly, this study showed that CD28 signaling caused a sustained inactivation of I $\kappa$ B $\alpha$ . FK506, another inhibitor of calcineurin, blocked the activation of c-Rel in both B and T cells (118).

**5.8.3 NITRIC OXIDE** Two recent reports indicate that production of nitric oxide (NO) inhibits the activation of NF- $\kappa$ B in endothelial cells (161, 162). The rationale for such a study is that NO inhibits platelet adhesion and smooth muscle proliferation, and modulates leukocyte adhesion to the endothelium. Thus, the production of NO suppresses immune and inflammatory responses. NO inhibits the activation of NF- $\kappa$ B in response to treatment with TNF $\alpha$  (161). The second study showed that NO inhibits the activation of NF- $\kappa$ B and provided evidence that this is through the activation of I $\kappa$ B $\alpha$  (162). Thus NO appears to inhibit activation of NF- $\kappa$ B through a mechanism similar to that described for glucocorticoids (i.e. through the upregulation of I $\kappa$ B $\alpha$ ). Since NF- $\kappa$ B transcriptionally regulates the iNOS gene, production of NO may feed back to block its own production through the inhibition of NF- $\kappa$ B. Adding to the complexity, production of NO in lymphocytes reportedly leads to the activation of NF- $\kappa$ B (163).

**5.8.4 cAMP** As with glucocorticoids and other immunosuppressants, agonists that elevate cAMP inhibit IL-2 expression. This appears to occur through the inhibition of two factors that activate the transcription of IL-2 (164). One of these is NF- $\kappa$ B and the other is uncharacterized and is called the "TGGGC" factor. The inhibition of NF- $\kappa$ B may involve decreased synthesis of NF- $\kappa$ B subunits, and decreased degradation or upregulation of I $\kappa$ B (164, 165). Agonists that increase cellular cAMP levels may be important modulators of immune responses. Prostaglandin E<sub>2</sub>, an activator of cAMP, may modulate immune function by inhibiting IL-2 secretion on the one hand and activating IL-4 and IL-5 on the other. Thus activators of cAMP may play an important role in the differentiation of TH cells into either the TH1 or TH2 category (164, 165).

## 6. NF- $\kappa$ B AND DISEASE

Because of its direct role in regulating responses to inflammatory cytokines and endotoxin, the activation of NF- $\kappa$ B may play a role in the development of chronic diseases such as rheumatoid arthritis or in acute situations such as septic shock. Support for a critical role for NF- $\kappa$ B activation in arthritis comes from two observations: NF- $\kappa$ B is activated in the arthritic synovium (S Makarov, A



Baldwin, unpublished), and therapies that are used for treatment, such as prednisone (see above) and gold compounds (166), are now known to block NF- $\kappa$ B.

Septic shock is a systemic inflammatory response that develops when LPS or other microbial products stimulate expression of various inflammatory cytokines. The massive production of these proteins ultimately leads to reduction in blood pressure and to general organ failure. It has recently been proposed that the production of nitric oxide in response to LPS regulates important aspects of septic shock (see 167). These experiments showed that iNOS-deficient mice were protected from septic shock. Since NF- $\kappa$ B activates transcription of the iNOS gene (see above), activation of NF- $\kappa$ B by LPS may play a role in the development of septic shock. Other activators of NF- $\kappa$ B, such as TNF $\alpha$ , may also mediate septic shock (see 168). Autoimmune diseases such as systemic lupus erythematosus (SLE) may involve activation of NF- $\kappa$ B as well (W Jarjour, personal communication). Additionally, Alzheimer's disease may also involve activation of NF- $\kappa$ B in a chronic setting, since the amyloid  $\beta$  peptide causes production of ROIs and activates gene expression through  $\kappa$ B sites (169). As described previously, NF- $\kappa$ B plays an important role in the activation of HIV gene expression. The influenza virus protein hemagglutinin activates NF- $\kappa$ B, and this activation may contribute to viral induction of cytokines and to some of the symptoms associated with flu (170). Other examples involving NF- $\kappa$ B and disease pathogenesis are briefly discussed below.

## 6.1 *Atherosclerosis*

Initiation and progression of atherosclerosis is related to the oxidation of lipids in LDLs. These oxidized lipids become trapped in the extracellular matrix of the subendothelial space and apparently activate NF- $\kappa$ B, leading to transcriptional activation of genes involved in the inflammatory process (171). This process leads to the development of the fatty streak associated with atherosclerosis. Interestingly, mice that are susceptible to atherosclerosis exhibit activation of NF- $\kappa$ B when fed an atherogenic diet (172). Another important contributor to atherosclerosis is thrombin, a serine protease that serves several important roles in actions upon inflammatory cells as well as cells of blood vessel walls. Thrombin likely contributes to the proliferation of the vascular wall that occurs in atherosclerosis and restenosis. Thrombin stimulates the proliferation of vascular smooth muscle cells through the activation of NF- $\kappa$ B (173). Overall, these data provide substantial evidence that NF- $\kappa$ B activation is an important contributor to events involved in atherosclerosis.

## 6.2 *Oncogenesis*

Evidence for involvement of NF- $\kappa$ B or I $\kappa$ B members in oncogenesis is based on several observations (4, 5): (i) NF- $\kappa$ B proteins are members of a proto-

oncogene family; (ii) the NF- $\kappa$ B2 gene and the *Bcl-3* gene are translocated in certain lymphomas; (iii) NF- $\kappa$ B is activated in quiescent fibroblasts in response to serum growth factors; (iv) NF- $\kappa$ B is activated by viral transforming proteins (Tax and LMP-1, for example; see above); and (v) exposure of cells to I $\kappa$ B $\alpha$  antisense results in oncogenic transformation (174). Additionally, antisense to RelA blocked tumorigenesis induced by Tax in vivo (175). Evidence for a role of translocated NF- $\kappa$ B2 in oncogenesis is based on observations that the normal repressive function of p100 is lost when the ankyrin repeats are deleted in the translocated allele (176, 177). A role for NF- $\kappa$ B/Rel proteins in human cancer presumably would involve transcriptional functions, such as the upregulation of the *c-myc* gene (see above). Conversely, it has been proposed that the activation of NF- $\kappa$ B in macrophages by the antineoplastic agent taxol may contribute to the compound's antitumor properties (178).

### 6.3 *Ataxia Telangiectasia*

Ataxia telangiectasia (AT) is a human disease characterized by neurological, radiobiological, and immunological deficiencies. Fibroblasts from AT patients are extremely sensitive to ionizing radiation, exhibiting aberrant regulation of DNA synthesis. Recently, a truncated form of I $\kappa$ B $\alpha$  (presumably functioning as a “super-repressor” based on the loss of the critical N-terminal serines) was shown to protect AT cells from killing by ionizing radiation and to correct the defect in DNA synthesis (179). These AT cells express constitutive levels of an NF- $\kappa$ B-like activity. Expression of the truncated form of I $\kappa$ B $\alpha$  reduced NF- $\kappa$ B levels and blocked the radiobiological effects. The gene involved in AT was recently cloned, and the encoded protein is related to yeast lipid kinases TOR1 and TOR2 as well as mammalian PI-3 kinase (180). An additional homology exists with the yeast RAD3 protein, which is involved in cell cycle control. It is proposed that the mutations in the AT gene render it nonfunctional. It is unclear whether NF- $\kappa$ B is activated in other cell types of AT patients and, if so, whether NF- $\kappa$ B may contribute to the neurological or immunological deficiencies. An interesting connection exists between AT patients and the I $\kappa$ B $\alpha$  gene knockout mice (89) in that both exhibit small spleens and thymuses. Thus, inappropriate activation of NF- $\kappa$ B in both cases could cause an immunological defect. The mechanism that relates the loss of function of a PI-3-like kinase/Rad3-like protein to activation of NF- $\kappa$ B is presently unclear.

## 7. SUMMARY

Ten years of research on NF- $\kappa$ B has led to a much greater understanding of the role of this family of transcription factors and their inhibitors in immunity, inflammation, and cell growth and development. The next challenge is to

untangle the signal transduction pathways involved in controlling activity of NF- $\kappa$ B and to uncover new gene targets for these proteins in immunological and nonimmunological function. Cell type-specific functions for NF- $\kappa$ B, for example in neurons, should be pursued. The functional roles of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ , as well as potential new forms of inhibitors, need to be clearly delineated. Additionally, it will be important to understand the exact roles for NF- $\kappa$ B in regulating apoptosis in several situations. Clarification of the mechanisms through which dysregulation of NF- $\kappa$ B contributes to disease is clearly needed. This could promote the development of specific inhibitors of NF- $\kappa$ B and thereby assist in treating certain of these diseases.

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